Supplementary Note

Optimization of CIRCLE-seq

To achieve restriction-enzyme independent circularization of genomic DNA, we tested a strategy based on ligation of an uracil-containing stem loop adapter to an end-repaired, A-tailed PCR amplicon. We enzymatically selected for covalently-closed DNA molecules that had stem-loop adapters ligated to both sides with a mixture of Lambda exonuclease and *E. coli* exonuclease I. 4 bp overhangs were released using a mixture of USER enzyme and T4 PNK, ligation was performed with T4 DNA ligase under conditions favoring intramolecular ligation, and successful circularization was measured by capillary electrophoresis (**Supplementary Fig. 3**). The conditions resulting in highest circularization efficiency (400 U T4 DNA ligase, 2.5 ng/ul DNA concentration) were used for circularization in all subsequent CIRCLE-seq experiments.

To determine which concentration of Cas9 ribonucleoprotein complex could fully cleave a PCR amplicon containing the corresponding gRNA target site *in vitro*, we performed *in vitro* cleavage assays at varying RNP concentrations. We found that near-complete cleavage of the target amplicon was achieved only with the highest concentration (90 nM Cas9, 9 nM DNA) (**Supplementary Fig. 12**).

We subsequently conducted CIRCLE-seq on two target sites at 4 protein concentrations and found that CIRCLE-seq remains sensitive even with these lower concentrations of nuclease, though the total number of off-target sites is reduced. However, one off-target site previously detected by GUIDE-seq was not identified in these lower concentration experiments, suggesting that CIRCLE-seq at the higher protein concentration is likely to yield the most comprehensive

results (**Supplementary Fig. 12**). This 10:1 RNP:DNA ratio was used for all other CIRCLE-seq experiments described.

To characterize the technical reproducibility of CIRCLE-seq, we performed independent library preparations from the same source of U2OS genomic DNA. We observed strong CIRCLE-seq read count correlations in independent technical replicates (**Supplementary Fig. 4**).

CIRCLE-seq on Repetitive Target Sites

To provide a more challenging test of CIRCLE-seq, we also profiled SpCas9 with four additional gRNAs targeted to repetitive sequences that had also been previously characterized by GUIDE-seq. Due to the repetitive nature of their targets, these four gRNAs have a relatively larger number of closely matched sites in the human genome (**Supplementary Table 1**) and, not unsurprisingly, have had been shown by GUIDE-seq to induce a large number of off-target effects in human cells[30]. As expected, CIRCLE-seq also identified a much larger number of off-target sites, ranging in number from 496 to 2503 for each of the four gRNAs (**Supplementary Table 2**) and distributed throughout the human genome. Included among these were 353 of the 364 off-target sites previously identified by GUIDE-seq experiments (**Supplementary Fig. 8**). For 9 of the 11 sites found by GUIDE-seq but not identified by CIRCLE-seq, evidence of supporting reads could be found in the CIRCLE-seq data but not of a sufficiently high number to meet our statistical threshold, once again suggesting that greater sequencing read depth should would enable detection of these sites.

Supplementary Table 1. Table of numbers of *in silico* off-target sites predicted in the human genome.

Target Site Sequence	Targetsite	0	1	2	3	4	5	6	7	8
GAGTCCGAGCAGAAGAAGAANGG	EMX1	1	1	2	27	421	4313	34761	218047	1156729
GGAATCCCTTCTGCAGCACCNGG	FANCF	1	1	3	33	449	3155	21793	135144	724696
GTCATCTTAGTCATTACCTGNGG	RNF2	1	1	1	11	204	2029	18023	138077	830825
GGGAAAGACCCAGCATCCGTNGG	Site_1	1	1	2	14	132	1499	13410	99120	627262
GAACACAAAGCATAGACTGCNGG	Site_2	1	1	2	16	239	3075	27129	180822	1026201
GGCCCAGACTGAGCACGTGANGG	Site_3	1	1	2	16	156	1831	15689	112679	645364
GGCACTGCGGCTGGAGGTGGNGG	Site_4	1	1	10	125	1231	9452	56139	297118	1471381
GGGTGGGGGGAGTTTGCTCCNGG	VEGFA_site_1	1	2	6	51	442	3870	28723	178630	929570
GACCCCCTCCACCCCGCCTCNGG	VEGFA_site_2	1	1	10	58	726	7636	51673	305299	1469770
GGTGAGTGAGTGTGTGCGTGNGG	VEGFA_site_3	1	2	37	1077	24857	530932	921004	1538579	2944099

Supplementary Table 2. List of all CIRCLE-seq detected off-target sites.

See attached file.

Supplementary Table 3. List of CIRCLE-seq read counts and HTGTS scores for off-target sites detected for Cas9 and gRNAs targeted against *EMX1* and *VEGFA* site 1.

See attached file.

Supplementary Table 4. Deep sequencing read counts for targeted tag integration sequencing of off-target cleavage sites of Cas9 and gRNAs targeted against *EMX1* and *VEGFA* site 1.

See attached file.

Supplementary Table 5. Listing of cell-type specific SNPs in protospacer or PAM of off-target cleavage sites detected by CIRCLE-seq.

See attached file.

Supplementary Table 6. Primers used in target tag integration sequencing.

See attached file.